FETUIN-A IS A POSSIBLE BIOMARKER OF DISEASE ACTIVITY IN MULTIPLE SCLEROSIS

MSRCNY

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INTRODUCTION

Previously, cerebrospinal fluid (CSF) proteomic analysis revealed elevation of Fetuin-A (Alpha2-Hermans-Schmid glycoprotein) in patients with multiple sclerosis (MS). Fetuin-A protein is of hepatic origin and is involved in a variety of biological functions such as regulation of calcium metabolism and osteogenesis, opsonization, and immune regulatory function. We investigated whether CSF Fetuin-A levels correlated with disease activity. MS brain tissue as well as brain and spinal cord from EAE mice were also analyzed to assess if elevated Fetuin-A levels correlated with neuropathological findings.

DESIGN AND METHODS

Sample Selection for ELISA Analysis

A total of eighty four CSF samples were obtained from patient volunteers at the International Multiple Sclerosis Management Practice (IMSMP) through an IRB-approved protocol. CSF was obtained from 43 patients with active MS and 41 patients with stable disease. Samples were collected by the side port of Medtronic pumps or lumbar puncture after informed consent was given by patients. All samples were coded and CSF aliquots were frozen at -70°C until analysis. Disease activity was determined by retrospective chart reviews of clinical and MRI findings. Active disease in MS was defined by three parameters: (1) one or more relapses in the past 6 months; (2) change in one point or greater in EDSS (Expanded Disability Status Scale) score in the past 6 months; and (3) change in MRI, specifically a change in the number or size of lesions or the presence of gadolinium enhancing lesions in the past 6 months. Six patients were excluded from analysis due to a variety of comorbid conditions that effect serum protein levels, such as myocardial infarction (1), cancer (1), pneumonia (1), hepatitis (1), steroid treatment (1), and intrathecal methotrexate treatment (1).

Fetuin-A protein ELISA Analysis

Levels of Fetuin-A in the CSF were determined using the human Fetuin-A ELISA kit according to the manufacturer's instructions. ELISA plates were measured spectrophotochemically at 450nm. The analytical limit of detection of Fetuin-A is <1ng/ml. Concentration of diluted samples were then read off the standard curve constructed by plotting the absorbance values against each respective human Fetuin-A standard level using a four-parameter function.

MS and Normal Brain Tissue

Fetuin-A protein expression was examined in 22 MS brain samples in plaques, and from areas of normal appearing white and grey matter. Ten control brains were similarly examined. Brain tissue specimen acquired from the Human Brain and Spinal Fluid Resource Center sponsored by NINDS/NIMH, National Multiple Sclerosis Society, Department of Veterans Affairs (Los Angeles, CA).

Induction and Evaluation of EAE

The protocol for induction of EAE in 15 C57BL/6 mice with myelin oligodendrocyte glycoprotein (MOG) peptide fragment 35-55 (Synthetic Biomolecules, CA) was followed as described in Stromnes, I. M. and Goverman, J. M. EAE Clinical score was assessed on a 0 to 5 scale as follows: grade 0, normal; grade 1, tail paralysis; grade 2, tail paralysis and hind-limb weakness (waddling gait); grade 3, hind limb paralysis; grade 4, hind limb plus forelimb paralysis; grade 5, moribund state. Spinal cords of C57BL/6 mice were obtained at day 28 after EAE induction at the peak of disease when the EAE score was between 3 and 4 and used for immunohistochemical staining. Five control mice were studied for comparison.

Immunohistochemistry

Immunohistochemical staining was performed using the avidin/biotin method on frozen and paraffin-embedded tissue sections (5µm). Briefly, after deparaffinization and rehydration, sections were blocked in 1X PBS/10% horse serum for 1 hour at room temperature and incubated with polyclonal anti-human (R&D Systems and Biovendor Laboratories) or anti-mouse Fetuin-A antibody (Santa Cruz Biotechnology Inc.) for 16 hours at 4°C. A biotinylated secondary antibody coupled with streptavidin-horseradish peroxidase was then used with 3,3-diaminobenzidine tetrahydrochloride (DAB) as a substrate. For myelin basic protein (MBP, Chemicon International) staining, appropriate anti-human and anti-mouse antibodies were used. Luxol fast blue (LFB) staining using standard protocol was used in paraffin-embedded human and mouse tissue. Hematoxylin and eosin (H&E) were used as routine nuclear and cytoplasmic counterstains. Positive and negative controls were included with each experiment.

RESULTS

CSF Analysis

The characteristics of the 84 MS patients and mean Fetuin-A levels of active and stable disease patients are shown in Table 1 and Figure 1, respectively.

Table 1. Patient Demographics

Determinants	Active Disease	Stable Disease
Number	43	41
F/M (ratio)	26/15 (1.73)	31/10 (3.1)
Mean Age	48	52
Age Range	22-74	33-72
Disease Duration	3-25	5-35
RRMS	10	10
SPMS	24	21
PPMS	9	10
Mean CSF Fetuin-A levels (ng/ml)	1778+-845.6	1300+-636.2
	P<0.01 (Mann-Whitney test)	

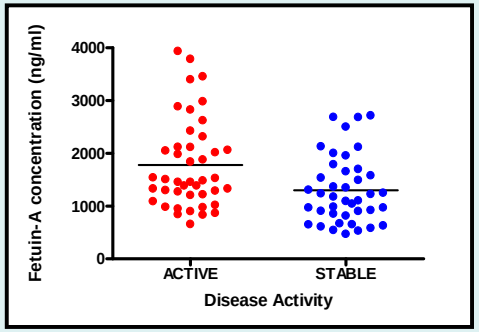

Figure 1. Scatter plot graph showing Fetuin-A protein levels measured by ELISA are upregulated in the CSF of MS patients with active over inactive disease (p=0.0059).

CSF levels of Fetuin-A were significantly elevated in patients with active disease in comparison to levels seen in stable disease across MS types. In RRMS, active disease patients had a mean value of CSF Fetuin-A levels ~800 ng/ml greater than patients with stable disease. By contrast, in patients with SPMS, where disease activity is less clearly definable, "active" disease patients had Fetuin-A levels of ~150 ng/ml greater than "stable" patients. Further, stable SPMS patients had mean Fetuin-A levels ~600 ng/ml higher than stable RRMS patients. This suggests that a low level of disease activity is always present in progressive forms of MS.

Human MS Brain Fetuin-A Immunostaining

By immunohistochemistry, Fetuin-A expression was increased in all MS plaques examined. The areas of increased Fetuin-A (**Figure 2A**) coincided with the areas of demyelination (**Figure 2B**). Similar results were found by MBP staining (not shown). By contrast, Fetuin-A in normal appearing white and grey matter of MS brains as well as in control brains, was not increased above background levels (not shown).

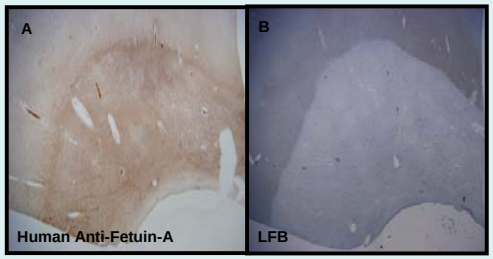

Figure 2. Increased Fetuin-A immunostaining in areas of demyelination in MS brain as viewed by 2X light microscopy.

Fetuin-A Immunostaining in EAE

Fetuin-A immunostaining in EAE mice spinal cords correlated strongly with areas of acute inflammation and demyelination (**Figure 3A-C, arrows**). By contrast, immunostaining for Fetuin-A was not detected in unaffected spinal cord tissue of EAE mice or in control mice spinal cord (not shown). LFB and H&E staining of EAE mice spinal cord show demyelination (**Figure 3A**) and cellular infiltration (**Figure 3B**) in active plaque areas that correlate with increased Fetuin-A staining in transverse sections of the same areas (**Figure 3C**). EAE mouse spinal cord section stained with only secondary antibody (**Figure 3D**) as a control.

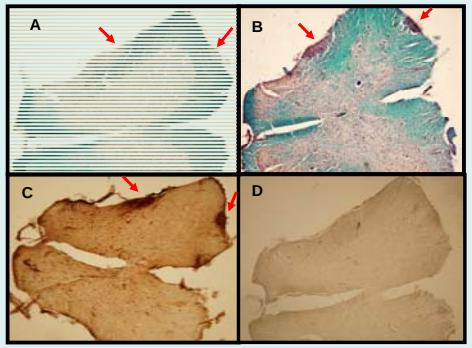

Figure 3: Increased Fetuin-A immunostaining in active EAE plaques as viewed by 10X light microscopy.

CONCLUSIONS

- CSF Fetuin-A levels are significantly elevated in patients with active disease by comparison to the levels seen in stable MS patients.
- By immunohistochemistry, Fetuin-A is specifically increased in MS plaques.
- In EAE mice, Fetuin-A is increased in areas of actively demyelinating disease.
- These novel findings suggest that CSF Fetuin-A levels may represent a biomarker of active disease.
- The pathophysiologic significance of our findings remains to be determined. The correlation with disease activity of Fetuin-A levels may be explained on the basis of its described functions. Fetuin-A is known to activate metalloproteinases and may result in enhanced matrix turnover and increased blood-brain barrier permeability. Also, Fetuin-A is an antagonist of TGF-β and thus may promote inflammatory activity.

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